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PATENT REQUEST : STANDARD PATENT

I, being the person identified below as the Applicant, request the grant of a Standard Patent to the person identified below as the Nominated Person, for an invention described in the accompanying complete specification.

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Invention Title: MAMMAL CELL LINES AND METHOD OF OBTAINING
GLYCOPROTEINS

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The invention relates to mammalian cell lines, particularly hamster cell lines which stably express glycoproteins 250 (220), 350 and 250 (220)/350 as Epstein-Barr virus antigens as well as to a process for obtaining these glycoproteins from cells cultivated in serum-free medium.

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**COMPLETE SPECIFICATION
STANDARD PATENT**

Applicant(s):

Hans WOLF

Invention Title:

MAMMAL CELL LINES AND METHOD OF OBTAINING GLYCOPROTEINS

The following statement is a full description of this invention, including the best method of performing it known to me/us:

Mammalian cell line and process of obtaining glycoproteins

The Epstein-Barr virus causes infectious mononucleosis in humans, one of the most frequent infectious diseases of young people.

More than 90% of the adult population has been infected with Epstein-Barr virus. These EBV positive people may later develop diseases such as nasopharyngal carcinomas, B cell lymphomas, T cell lymphomas, certain Hodgkin lymphomas, and possibly other malignant diseases. In direct time relation to the primary infection or at a later stage chronic forms and in rare cases even acute lethal forms have been reported.

Since an infection with Epstein-Barr virus in small children remains clinically inconspicuous, and since vaccination with recombinant vaccine viruses that express the Epstein-Barr virus membrane antigen gp 250/350 (BLLF-1 reading frame, Baer et al., 1984) confers protection against infection and/or diseases (Gu et al., 1993), it must be assumed that a vaccine against Epstein-Barr virus related diseases is possible.

Due to the possible side-effects of live vaccines the development of pure protein vaccines is a major target. Since the Epstein-Barr virus membrane protein gp 250/350 (BLLF-1) is highly glycosylated and this posttranslational modification is an important component of correct immunogenicity (Emini et al., 1988), the development of a vaccine on the basis of a glycoprotein producible in eucaryotic cells on the basis of gp 250/350 (reading frame BLLF-1 of Epstein-Barr virus) seems very promising.

The invention relates to mammalian cell lines, particularly hamster cell lines that stably express the glycoprotein (gp 250 (220)), the glycoprotein (gp 350) and/or the hybrid glycoprotein (gp 250 (220)/(gp) 350) as Epstein-Barr virus antigens, particularly in a protein-free medium, thereby avoiding the risk of a viral or bacterial contamination through medium supplements. The invention furthermore relates to a process for the production of said glycoproteins.

Hamster cell lines that stably express gp 250 (220), gp 350 and/or gp 250 (220)/gp 350 as EBV antigens are known; cf., e.g., Motz et al. in Gene, 44 (1986), 353-359 and Vaccines, 87 (1987), 374-379, as well as Gu Shyan et al. in 100 Jahre Blutserum-Therapie 1890-1990, Helle (Saale), (1991), 93-100. To date, however, no hamster cell lines have been known with which said glycoproteins can be stably expressed. According to Motz et al. in Vaccines 87 (1987), 374-379, 376 the integration of the viral foreign glycoproteins in CHO cell membranes can be toxic beyond a certain point.

According to one embodiment of the invention a hamster cell line was provided by following a specific genetic strategy which cell line stably expresses and secretes said glycoproteins in large amounts and which is obtainable by

- transfecting the cell line with a vector expressing said glycoproteins but not the membrane anchor (Motz et al., 1987),
- wherein the vector comprises a selection marker which is absent from the cell line,
- cultivating the cell line and selecting cells with the help of a selection marker, which cells stably express and secrete the glycoproteins once the selection factor (inhibitor) is removed.

A further embodiment provides the use of a process for producing a hamster cell line that stably expresses said glycoproteins in a protein-free medium and that is obtainable by

- (A) starting from a hamster cell line (host cell line) which is devoid of a selection marker which is later exhibited by a vector to be chromosomally integrated according to (A) (d) or a vector to be chromosomally integrated according to (B) (recombinant cell line),
 - (a) cultivating the cell in a serum-containing medium and allowing them to adhere to the substrate and to one another,
 - (b) repeatedly exchanging part of the consumed medium, gradually reducing the serum content of the medium while so doing and finally cultivating it serum-free, thereby
 - not actively removing adherence of the cells and
 - optionally allowing cell spheroids to form and to detach, separating the detached cell spheroids, cultivating them in suspension while gently shaking them and selecting cells that grow in small aggregates or singly and
 - (c) finally subjecting the selected cells to the process steps according to claim 1; or
- (B) subjecting a hamster cell according to claim 1 to the process steps (A) (a) to (A) (b).

The starting cell line or the host cell line is allowed to grow in a serum-containing culture medium, e.g., in MEM alpha- or a- + 5% FCS since this cell line cannot yet be transferred in media nor cultivated in media that are serum-free. Examples for such culture media are DIF 1000, RPMI 1640, ASF 103 and HDB.

There are three aspects that are relevant in order to obtain growth in protein-free culture medium.

1. The adherence during serum depletion of the culture is made use of while working, e.g., with culture flasks. The result of this procedure is a positive growth stimulation. Furthermore, old consumed medium and dead cells as well as products of a lysis can be separated easily and very gently (without centrifugation).
2. Old medium is repeatedly exchanged only partially so as to guarantee a good self-conditioning of the medium during serum depletion.
3. The cells are subjected to a long-term cultivation with gentle mixing, e.g., in spinner flasks in suspension culture whereby spheroids are formed. Specific selection for reduced adherence tendency allows to isolate cells that grow in small aggregates (spheroids) or singly. First, big spheroids are separated. Then the supernatant is subjected to fractional centrifugation at low g values and the separated small spheroids and single cells are used for further passaging.

Growth is possible in the following protein-free medium when using the strategy set forth in claim 2. The medium is referred to hereinafter as SMIF (Scharfenberg's Modified Iscove's F12 Medium). It is a well enriched medium, consisting of a mixture of about 1:1 of Iscove's Modified Dulbecco's Medium with Ham's F12 nutrient (IF), to which, e.g., Putrescin (e.g., 1.2 micromol l^{-1}) and L-hydroxyprolin (e.g., 153 micromol l^{-1}) (SMIF1) can be added. Furthermore, for a growth in suspension chelating agents should preferably be added such as aurintricarboxylic acid (ATA, e.g., 3 micromol l^{-1}) (Bertheussen, 1993), EDTA (e.g., 4.0 micromol l^{-1}) and citric acid (e.g., 40 micromol l^{-1}) for

complexing divalent ions and for an improved availability of inorganic iron as substitute for the protein component transferrin which is usually employed in serum-free media (SMIF2).

5 According to a specific embodiment of the invention hamster cell lines are obtainable by starting from a Chinese Hamster Ovary cell line (CHO), e.g., CHO-K1 = ATCC CCL 61, or from a Baby Hamster Kidney cell line (BHK), e.g., BHK 21C13 = ATCC CCL 10.

10 If it is possible according to the invention to generate hamster cell lines with generation times of, e.g., 15 to 40 and particularly of 20 to 30 hrs, with which the above-mentioned glycoproteins can be stably expressed, this achievement must be considered inventive in that to date 15 nobody has succeeded in cultivating hamster cell lines in protein-free medium without selective genetic manipulation (genetic engineering). To date, it has been believed that mammalian cell lines must be prepared for growth in protein-free medium by genetic engineering so as to express 20 the desired gene products (NSO cell line, Hassel et al., 1992).

For selection markers and inhibitors the skilled practitioner can rely on the relevant prior art. An example of a suitable selection marker is the dihydrofolate 25 reductase gene (DHFR gene) and an example of a suitable inhibitor is methotrexate (MTX) (Motz et al., 1987).

A specific embodiment of the invention is the hamster cell line CHO pMDIIIGPTR-PFC6, a sample of which was deposited under the provisions of the Budapest Treaty 30 on 15 March 1993 at the DSM-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH and which has been accorded accession no. DSM ACC 2121.

gp 250 (220), gp 350 and/or gp 250 (220)/gp 350 are obtainable according to the invention by 35 (a) cultivating a cell line according to the invention and allowing the desired glycoproteins to be expressed,

- (b) separating the cells from the culture medium, particularly by microfiltration,
- (c) subsequently concentrating and purifying the clear crude supernatant by ultrafiltration, particularly by cross-flow ultrafiltration,
- (d) subsequently purifying it using an anion exchange column,
- (e) ultrafiltration and
- (f) gel filtration and obtaining the resulting fractions of pure glycoproteins (gp).

According to the process of the invention the glycoproteins can be obtained in native and glycosylated form. They thus possess their natural antigenic potential. The vaccine obtained by this method thus exhibits antigenic properties that correspond to those of the respective proteins of the active EB virus. The vaccine obtained is a safe vaccine having a high potential of antigenic protection without having pathogenic potential. The recombinant vaccine is therefore superior to an inactivated or weakened vaccine in terms of safety since neither a potential for reversion of the weakened viruses nor the minimal risk and the toxicology of inactivated viruses must be expected.

Example 1: Obtaining CHO pMDIIIGPTR-PFC6

A clone derived from cell line CHO K1 = ATCC CCL61 which lacks the dihydrofolate reductase gene (dhfr-1) was transfected with plasmid pMDIIIGP according to Motz et al. in Gene, 44 (1986), 353-359; Motz et al. in Gene, 58 (1987), 149-154 and Vaccines, 87 (1987) 374-379. The cells were cultivated and clones were selected with methotrexate (MTX) which stably express gp 250/350 even if no inhibitor is used. A clone with stable expression of gp 250/350 was referred to as CHO C6.

Example 2: Cultivation of CHO C6 in protein-free medium

Cultivation was performed under standard cell culture conditions. CHO C6 was cultivated in IF medium containing 5% fetal calf serum (FCS) in 1 to 2 big culture flasks (185 cm², about 40 to 50 ml volume; Falcon) up to absolute cell confluence. In some cases multilayers already formed. Subsequent dilution of FCS was done gradually via an FCS content of 1, 0.2, 0.04 and 0.008%, by replacing each time 4/5 of the old medium including live and dead free cells as well as lysates by fresh, preheated SMIF1. If free spheroids were formed in the culture at low FCS contents, they were able to settle before the medium was removed. The time of the exchange depended on the vitality and the condition of the culture and was determined individually, at the latest when the culture supernatant contained too low amounts of nutrients. A good standard value for an exchange is the glucose content of about 0.7 to 1 g/l. The medium was exchanged at least after a week at a lower share so as to supplement disintegrated nutrients.

When the medium was consumed at an FCS content of 0.008%, the entire medium was replaced by fresh protein-free preheated SMIF1 and further cultivated in culture flasks. If under these conditions already a sufficient amount of spheroids was formed or detached, these spheroids were transferred to a small spinner culture flask (40 ml minimum volume) and cultivated in 40 ml SMIF1 (1/4 old conditioned plus 3/4 fresh medium) at about 40 rpm. If the cells did not detach, the cell layer had to be enzymatically isolated from the substrate such as is usually done for passaging adherent cells. The cells that easily aggregate in this condition were purified from protease after they had detached by twice washing them with SMIF1 medium before the cells could also be transferred to small spinner flasks.

The cells were cultivated in the spinner flask until generation times of less than 40 hrs were obtained and the culture grew in small spheroids. Passaging was done at a standard value of about 0.5 to 1 g remaining glucose/l in the medium. Small spheroids and single cells were selectively passaged. For this purpose, the cell culture suspension was not stirred for a short period so that large spheroids could detach. The supernatant (100 g) was then removed by centrifugation. The cells in the pellet were transferred to the next passage. The culture medium was conditioned by adding 20 to 30 vol.-% medium of the previous passage which before had been cleansed by filtration or centrifugation from dead cells and cell fragments.

The entire process for two adaptation attempts took more than two months each.

Example 3: Processing and purification of the recombinant proteins from culture supernatants

I. Cross-flow ultrafiltration followed by rebuffering

For this purpose the microfiltered harvests of fermentations in protein-free SMIF1 or SMIF2 were used, namely from continually perfused culture up to the 2 l scale or from batchwise culture in a 10 l airlift fermenter. For filtration filter cassettes with a nominal cut off of 100 kD (Millipore or Filtron) were used. Standard values during cross-flow ultrafiltration were the following (relatively randomly chosen):

- transmembrane pressure not exceeding about 1 bar;
- about 1/3 of the crude supernatant present in the circuit to be filtered was removed from the volume flow as filtrate; this corresponded to about 2-3 l

filtrate for one filter cassette and a suction capacity of, e.g., 8 l/min;
- during the buffer exchange lower pumping capacities were used.

In general the following steps are provided. The first two steps can be dispensed with, however, they could improve the yield.

1. The overall harvest volume was determined and about 15 vol.-% filtrate were generated (based on the volume flows under normal filtration conditions) in order to condition the membrane, i.e., to adjust a constant effective filter cut off by loading and polarizing the membrane.
2. Said first filtrate was reunited with the retentate.
3. The actual cross-flow ultrafiltration was performed until the residue was reduced to about a 50th of the starting volume. However, it is possible to concentrate the residue even more, particularly by using protein-free medium.
4. The primary residue was buffer-exchanged and washed when the end volume was obtained. For this purpose the residue was replenished to the double volume three to four times (including the dead volume of the pump and ultrafiltration system) with phosphate buffer (20 mM; pH 7) and concentrated to the minimum residue and provided for further processing. This step serves the purpose of removing low molecular exogenous proteins and of reducing the ionic strength for the subsequent purification step.

II. Purification over an anion exchange column

The following equipment was used.

Column material: Q-Sepharose High Performance (a BioProcess material of Pharmacia)

Buffer system: buffer A: 20 mM phosphate buffer (pH 5.1)
buffer B: 20 mM phosphate buffer (pH 5.1) with 1 M NaCl

Scale: for example small scale with FPLC and HR 5/5 column having a 1 ml bed volume (Pharmacia); scale-up possible.

Flow rate: constant 5.1 cm/min corresponding to 1 ml/min in an HR 5/5 column

The ultrafiltration residue was diluted with MilliQ process water to a conductivity of 3 mS and adjusted with HCl to a pH of 5.1 (e.g., first to pH 4.2 in order to separate a higher share of exogenous proteins and then to pH 5.1). The resulting turbidity was removed by centrifugation. Then the column was loaded with the clear supernatant via an FPLC pump while adding a 5% portion of buffer B to elute a first exogenous protein. The maximum load was about 8 mg total protein/ml gel. After that the protein was eluted via a gradient to 0.4 M NaCl (gradient over 22 column volumes).

The desired gp 250/350 protein was eluted between 0.15 and 0.35 M NaCl and the exact choice of the fractions to be further purified was made after polyacrylamide gel electrophoresis (PAGE). It was possible to achieve

a protein load and separation also without adding buffer B and also at a different pH (e.g., pH 7). The lower pH as well as the addition of buffer B during loading increase the capacity with respect to the gp proteins during chromatography.

If phosphate buffer was used, the rebuffering step prior to the subsequent process steps could be dispensed with. However, other buffer systems such as citrate buffer are also useful.

III. Ultrafiltration

The selected and collected protein fractions of step II were concentrated on a gel filtration column by ultrafiltration by a factor of about 10 to 20. The protein end concentrations were in the range of, e.g., 1 to 2 mg/ml. Minicon CS15 chambers (Amicon) were used for ultrafiltration. (At larger solvent volumes correspondingly larger filtration devices having higher capacities should be considered, for instance, agitator cells or cross-flow ultrafiltration modules.)

IV. Purification by gel filtration

Column material: HR 10/30 prepacked columns with Superose 6 (Pharmacia); for a scale-up Sephadryl S-300 High Resolution and Sephadryl S-400 High Resolution in a XK 26/100 column (Pharmacia) was used.

Buffer system: isocratic 100 or 200 mM NaCl in a 20 mM phosphate buffer (pH 7).

Flow rate: e.g., 2-2.5 ml/min

After equilibration of the column, e.g., 10 g protein per ml gel bed were applied which were first concentrated (CS15) and from which turbidities were removed by centrifugation. It was eluted at a flow rate of about 2.5 mm/min and separated. Those elution fractions were collected that contained gp 250 and/or gp 350 without visible contaminations after PAGE and silver staining.

Pure gp 250/350 can be stably stored in collected gp fractions of the gel filtration in a frozen state at about -20°C. The purification process had an overall yield of about 25-40% of pure crude product.

Comparative Example 1

Example 2 was repeated except that during the serum depletion stage no cell confluence was allowed and that FCS depletion was performed when cell adherence was abolished. With this process no cells cultivatable in protein-free medium were obtained.

The entire disclosure in the complete specification of our Australian Patent Application No. 67220/94 is by this cross-reference incorporated into the present specification.

Literature

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Hassel, T. et al. Stability of recombinant antibodies from glutamine synthetase amplified CHO and NSO cell lines. In: *Animal Cell Technology: Developments, Processes and Products* (Spier, R.E., Griffiths, J.B., MacDonald, C., eds.) Butterworths-Heinemann, Oxford, (1992) 42-47.

Motz M. et al. Truncated versions of the two major Epstein-Barr viral glycoproteins (gp 250/350) are secreted by recombinant Chinese hamster ovary cells. *Gene*, 58 (1987) 149-154.

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS.

1. Mammalian cell line which stably expresses glycoprotein (gp) 250 (220), glycoprotein (gp) 350 and/or the hybrid glycoprotein (gp) 250 (220)/(gp) 350 as Epstein-Barr virus antigens in a protein-free medium and which is obtainable by
 - (A) starting from a hamster cell line which is devoid of a selection marker which later on is exhibited by a vector to be chromosomally integrated according to
 - (A) (c) or a vector chromosomally integrated according to (B),
 - (a) cultivating the cells in a serum-containing medium and allowing them to adhere to the substrate and to one another,
 - (b) repeatedly exchanging part of the consumed medium, slowly reducing the serum content of the medium while so doing and finally cultivating it serum-free and
 - not actively removing adherence of the cells and
 - optionally allowing cell spheroids to form and to detach, separating the detached cell spheroids, cultivating them in suspension while gently shaking them and selecting cells that grow in small aggregates or singly and
 - (c) finally subjecting the selected cells to the following process steps
 - (ca) transfecting the cell line with a vector expressing the above-mentioned glycoproteins (gp), wherein the vector comprises a selection marker which is absent from said cell line,
 - (cb) cultivating the cell line and selecting cells with the help of the selection marker that stably express the glycoproteins once the

selecting factor (inhibitor) is no longer used, or

(B) subjecting a mammalian cell line first to the process steps (A)(ca) and (A)(cb) and then to the process steps (A) (a) to (A) (b).

2. The mammalian cell line according to claim 1, characterized in that a hamster cell line is used as the mammalian cell line, particularly a Chinese Hamster Ovary cell line (CHO) such as CHO K1 = ATCC CCL61, or a Baby Hamster Kidney cell line (BHK) such as BHK 21 C13 = ATCC CCL10.
3. The hamster cell line according to any of the preceding claims, characterized in that the dihydrofolate reductase gene (DHFR gene) is used as selection marker and methotrexate (MTX) as inhibitor.
4. Hamster cell line CHO-K1 pMDIIIGPTR-PFC6 = DSM ACC 2121.
5. Process for the production of glycoprotein (gp) 250 (220), glycoprotein (gp) 350 and/or the hybrid protein (gp) 250 (220)/(gp) 350, characterized by
 - (a) cultivating a cell line according to any of claims 1 to 4 and allowing it to express the desired glycoproteins and secrete them into the medium,
 - (b) isolating the glycoproteins from the medium.
6. Process for the production of glycoprotein (gp) 250 (220), glycoprotein (gp) 350 and/or the hybrid protein (gp) 250 (220)/(gp) 350, characterized by
 - (a) cultivating a cell line according to any of claims 1 to 4 and allowing it to express the desired glycoproteins and secrete them into the medium,
 - (b) separating the cells from the culture medium, particularly by microfiltration,

- (c) concentrating them by cross-flow ultrafiltration and purifying them,
- (d) afterwards purifying them with the help of an anion exchange column,
- (e) ultrafiltration and
- (f) gelfiltration and obtaining the resulting pure glycoprotein fractions (gp).

7. Vaccine containing glycoprotein (gp) 250 (220), glycoprotein (gp) 350 and/or the hybrid protein (gp) 250 (220)/(gp) 350 obtainable by the process according to claim 5 or 6.

8. Protein-free culture medium, consisting of an about 1:1 mixture of Iscove's Modified Dulbecco's Medium with Ham's F12 nutrient, additionally containing Putrescin and L-hydroxyprotein.

9. The culture medium according to claim 8, characterized in that the Putrescin is contained in a concentration of 1.2 $\mu\text{Mol/l}$ and the L-hydroxyprotein in a concentration of 153 $\mu\text{Mol/l}$.

10. The culture medium according to claim 8 or 9, characterized by additionally containing at least one chelating agent.

11. The culture medium according to claim 10, characterized in that at least one of the chelating agents is aurintricarboxylic acid, preferably in a concentration of 3 $\mu\text{Mol/l}$, EDTA, preferably in a concentration of 4.3 $\mu\text{Mol/l}$ or citric acid, preferably in a concentration of 40 $\mu\text{Mol/l}$.

Dated this 11th day of August 1998
HANS WOLF
By their Patent Attorneys
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Fellows Institute of Patent
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Abstract

The invention relates to mammalian cell lines, particularly hamster cell lines which stably express glycoproteins 250 (220), 350 and 250 (220)/350 as Epstein-Barr virus antigens as well as to a process for obtaining these glycoproteins from cells cultivated in serum-free medium.